

Role of parental folate pathway single nucleotide polymorphisms in altering the susceptibility to neural tube defects in South India

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Abstract

Aim: To investigate the role of four parental folate pathway single nucleotide polymorphisms (SNPs) i.e., methylene tetrahydrofolate reductase (MTHFR) 677C>T, MTHFR 1298A>C, methionine synthase reductase (MTRR) 66A>G and glutamate carboxypeptidase (GCP) II 1561C>T on susceptibility to neural tube defects (NTDs) in 50 couples with NTD offspring and 80 couples with normal pregnancy outcome.

Results: Maternal MTHFR 677C → T (odds ratio (OR): 2.69, 95% confidence interval (CI): 1.35–5.34) and parental GCP II 1561C → T (maternal: OR: 1.89, 95% CI: 1.12–3.21 and paternal: OR: 3.23, 95% CI: 1.76–5.93) were found to be risk factors for a NTD. Both paternal and maternal GCP II T-variant alleles were found to interact with MTHFR 677T- and MTRR G-variant alleles in increasing the risk for NTD. Segregation of data based on type of defect revealed an association between maternal 677T-allele and meningomyelocele (OR: 9.00, 95% CI: 3.77–21.55, $P < 0.0001$) and an association between parental GCP II 1561T-allele and anencephaly (maternal: OR: 2.25, 95% CI: 1.12–4.50, $P < 0.05$ and paternal: OR: 4.26, 95% CI: 2.01–9.09, $P < 0.001$).

Conclusions: Maternal MTHFR C677T and parental GCP II C1561T polymorphisms are associated with increased risk for NTDs. Apart from individual genetic effects, epistatic interactions were also observed.

Keywords: Glutamate carboxypeptidase (GCP) II; methionine synthase reductase (MTRR); methylene tetrahydrofolate reductase (MTHFR); neural tube defects (NTDS).

Introduction

High incidence of neural tube defects (NTDs) was observed in South India [8] where consanguinity is common [7] and vitamin deficiencies were reported [22], which indicate the role of genetic and nutritional factors as the possible etiological factors specifically pointing towards folate metabolism [9]. Hyperhomocysteinemia [12] and methylene tetrahydrofolate reductase (MTHFR, MIM No: *607093) C677T [19] and A1298C [18] polymorphisms are well-documented risk factors for NTD in other populations. Polymorphisms in cystathionine beta synthase (CBS, MIM No: +236200) [11], methyltetrahydrofolate homocysteine methyl transferase (MTR, MIM No: *156570) [3] and methionine synthase reductase (MTRR, MIM No: *602568) [18] and glutamate carboxypeptidase II (GCP II/FOLH1, MIM No: *600934) [14] were also studied for possible association with NTDs. However, data on GCP II are sparse. There are only two studies from India, one projecting paternal hyperhomocysteinemia and maternal folate deficiency as risk factors for NTD [12], whereas the other projecting MTHFR C677T genetic polymorphism as a risk factor for NTD [4]. Neural tube development occurs very early in fetal life prior to first noticeable signs of pregnancy and post-conceptual folate supplementation in such cases is not beneficial in reducing the risk for NTDs. In developed countries, fortification of foods with folic acid helped in reducing such risk whereas in India there is no such public health measure and the genetic risk factor profile might be different.

MTHFR is a rate-limiting enzyme in this pathway as its activity determines the proportion of 5, 10-methylene tetrahydrofolate and 5-methyl tetrahydrofolate available for thymidylate synthesis and cellular methylation, respectively. Two polymorphisms i.e., C677T and A1298C are widely distributed throughout the globe and reduce specific activity of MTHFR differentially. The former polymorphism induces a thermolabile variant enzyme whereas the later polymorphism acts synergistically when co-segregated with the former. GCP II, a folate hydrolase, hydrolyzes dietary folate (folyl polyglutamates) to monoglutamates thus assisting in intestinal absorption of folate. GCP II C1561T polymorphism impairs intestinal absorption of folate especially when the major source of folate is in polyglutamate form. MTRR is an enzyme which helps in regeneration of inactive MTR by reductive methylation of cobalamin. Cobalamin deficiency coupled with MTR A66G polymorphism might significantly impair re-methylation of homocysteine. The current study was aimed to explore the role of these four folate pathway

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genetic polymorphisms i.e., MTHFR C677T, MTHFR A1298C, MTRR A66G and GCP II C1561T in altering the susceptibility to NTD in a South Indian cohort.

Subjects and methods

Fifty couples with an offspring with NTD were enrolled as cases. The mean maternal and paternal age was 24.7 and 29.6 years, respectively. Parental consanguinity was observed in 8% cases: 6% were first cousins and 2% were uncle-niece marriages. These couples altogether had 103 pregnancies, 60 of which had a NTD. Information about the type of defect was available for all cases: 26 (43.4%) anencephaly, 6 (10%) encephalocele, 28 (46.6%) spina bifida [11 (18.3%) meningocele, and 17 (28.3%) meningomyelocele].

Eighty maternal and 80 paternal controls were recruited for this study. The mean maternal and paternal age was 24.3 and 28.9 years, respectively. Parental consanguinity was observed in 10% of controls: 7.5% were first cousins and 2.5% were uncle-niece marriages. On the average these controls had 2–3 pregnancies and all pregnancies were normal. No history of any miscarriage or pregnancy indicative of any congenital anomaly was observed. These controls were of same ethnic group as the cases.

Neither cases nor controls were on pre- and peri-conceptional folate supplementation of the indexed pregnancy. They have not used any fortified food.

Subjects with IDDM, hypertension, chromosomal anomalies and those on anti-folate medications were excluded from the study group.

Informed consent was obtained from all subjects and ethical clearance was obtained from the Ethical Committee of the Center for DNA Fingerprinting and Diagnostics, Hyderabad, India.

Whole blood samples in EDTA were obtained from all subjects and genomic DNA was isolated using standard protocols.

Genetic analyses

MTHFR 677C→T polymorphism A 173-bp band from exon 4 of MTHFR was amplified using specific primers i.e., 5'-TTT GAG GCT GAC CTG AAG CAC TTG AAG GAG-3' and 5'-GAG TGG TAG CCC TGG ATG GGA AAG ATC CCG-3'. Each 25 µL of PCR mixture was composed of 100 ng genomic DNA, 2.5 µL 10× PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH: 9.0), 1.5 mM Mg Cl₂], 0.2 mM each of deoxynucleoside triphosphate, 10 pmol of each primer and 1 unit of Taq DNA polymerase. The PCR conditions were: initial denaturation, 95°C for 5 min; denaturation, 95°C for 1 min; annealing, 60°C for 1 min; extension, 72°C for 1 min; and number of cycles, 30. To test for the polymorphism, 20 µL of PCR product was digested with 1 unit of HinfI restriction enzyme (recognition site: 5'-G[↓]ANTC-3') in 1X NEB buffer 2. Presence of MTHFR 677T-variant allele creates HinfI restriction site causing cleavage of 173-bp product into 125-bp and 48-bp fragments [6].

MTHFR A→C polymorphism A 163-bp band from exon 7 of MTHFR was amplified using specific primers 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3' and 5'-CAC TTT GTG ACC ATT CCG GTT TG-3'. Each 25 µL of PCR mixture was composed of 100 ng genomic DNA, 2.5 µL 10× PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH: 9.0), 2.5 mM Mg Cl₂], 0.2 mM each of deoxynucleoside triphosphate, 10 pmol of each primer and 1 unit

of Taq DNA polymerase. The PCR conditions were: initial denaturation, 95°C for 5 min; denaturation, 95°C for 1 min; annealing, 53°C for 1 min; extension, 72°C for 1 min; and number of cycles, 40. The PCR product was digested with *Mbo*II restriction enzyme (recognition site: 5'-GAAGA (N)⁸-3'). 1298C-variant allele abolishes one restriction site of *Mbo*II (84 bp, 31 bp, 30 bp, 18 bp) whereas 1298A allele causes further cleavage (56 bp, 31 bp, 30 bp, 28 bp, 18 bp) [18].

GCPII 1561C→T polymorphism A 244-bp band from exon 13 of GCP II was amplified using specific primers i.e., 5'-CAT TCT GGT AGG AAT TTA GCA-3' and 5'-AAA CAC CAC CTA TGT TTA ACA-3'. Each 25 µL of PCR mixture was composed of 100 ng genomic DNA, 2.5 µL 10× PCR buffer, 0.2 mM each of deoxynucleoside triphosphate, 10 pmol of each primer and 1 unit of Taq DNA polymerase. The PCR conditions were: initial denaturation, 95°C for 5 min; denaturation, To test for the polymorphism, 20 µL of PCR product was digested with 1 unit of AccI restriction enzyme in 1X New England Biolabs (NEB) buffer 4. Presence of GCP II 1561T-variant allele creates AccI restriction site (5'-GT[↓]MKAC-3') causing cleavage of 244-bp product into 141-bp and 103-bp fragments [5].

MTRR 66A→G polymorphism Restriction digestion analysis was performed with an artificially created NdeI restriction site using the sense primer: 5'-GCA AAG GCC ATC GCA GAA GAC AT-3' and antisense primer: 5'-CAC TTC CCA ACC AAA ATT CTT CAA AG-3', where the underlined C replaces the A to generate an NdeI restriction site (5'-CA[↓]TATG-3') in the normal sequence. Each 25 µL of PCR mixture contained 100 ng DNA, 2 µL PCR buffer, 0.2 mM each of deoxynucleoside triphosphate, 10 pmol of each primer, and 1 unit of Taq DNA polymerase. The PCR conditions were: initial denaturation, 95°C for 10 min; denaturation, 95°C for 1 min; annealing, 55°C for 30 s; extension, 72°C for 30 s; and number of cycles, 30. To test for the polymorphism, 20 µL of PCR product was digested with 2 units of NdeI in 1×NEB buffer 4. The PCR fragment of 66-bp remains uncut in the presence of the G (methionine) allele but is digested into fragments of 44-bp and 22-bp in the presence of the A (isoleucine) allele [14].

Statistical analyses

Univariate analysis was carried out taking genotype frequencies and allele frequencies as variables. Two-by-two contingency tables were prepared for cases and controls based on presence or absence of each variable. Frequencies, odds ratios (OR) and 95% confidence intervals (CI) were calculated using Vassarstats software (<http://faculty.vassar.edu/lowry/VassarStats.html>). Fisher exact test was performed to obtain P-values. All statistical tests were based on two-tailed probability and considered significant at P<0.05. Normal, heterozygous and homozygous mutant genotypes were labeled as 0, 1 and 2, respectively based on number of mutated alleles and logistic regression analysis was carried out to obtain P trend values. P HWE values were obtained by χ^2 -test for the observed frequencies and expected frequencies according to Hardy–Weinberg equilibrium test.

Bivariate analysis was done by computing the haplotype data in -/-, -/+, +/-, +/+ format where “-” indicates absence and “+” indicates presence of the mutated alleles. For pair-wise analysis, Fisher exact test was employed on each haplotype. Logistic regression analysis for the four different haplotypes in cases and

controls was done to explore the possibility of gene-gene interaction.

Results

Role of individual polymorphisms

Table 1 represents the distribution of genotypes and alleles for individual polymorphisms in case and control couples.

MTHFR 677C→T Out of 50 NTD case mothers, 33 (66%) were normal, 11 (22%) were heterozygous and 6 (12%) were homozygous mutant for MTHFR 677C→T polymorphism. Out of 80 control mothers, 64 (80%) were normal, 16 (20%) were heterozygous and none were homozygous mutant. The 677T-allele frequencies were 23% and 10% in NTD case mothers and control mothers, respectively. This variant allele was associated with 2.7-fold risk for NTD.

Out of 50 NTD case fathers, 43 (86%) were normal, 7 (14%) were heterozygous and none were homozygous mutant for this polymorphism. Out of 80 control fathers, 72 (90%) were normal, 8 (10%) were heterozygous and none were homozygous mutant. The 677T-allele frequencies were 7% and 5% in NTD case fathers and control fathers, respec-

tively. This variant allele was not associated with any significant risk for NTD.

MTHFR 1298A→C Out of 50 NTD case mothers, 22 (44%) were normal, 25 (50%) were heterozygous and 3 (6%) were homozygous mutant for MTHFR 1298A→C polymorphism. Out of 80 control mothers, 26 (32.5%) were normal, 40 (50%) were heterozygous and 14 (17.5%) were homozygous mutant. The 1298C-allele frequencies were 31% and 42.5% in NTD case mothers and control mothers, respectively. This variant allele was not associated with any significant risk for NTD.

Out of 50 NTD case fathers, 8 (16%) were normal, 36 (72%) were heterozygous and 6 (12%) were homozygous mutant. Out of the 80 control fathers, 26 (32.5%) were normal, 40 (50%) were heterozygous and 14 (17.5%) were homozygous mutant. The 1298 C-allele frequencies were 48% and 42.5%, respectively in NTD case fathers and control fathers, respectively. This variant allele was not associated with any significant risk for NTD.

GCP II 1561C→T Out of 50 NTD case mothers, 9 (18%) were normal, 41 (82%) were heterozygous and none were homozygous mutant for GCP II 1561C→T polymorphism.

Table 1 Genotype/allele frequency distribution in couples with NTD and in couples with normal reproductive history.

SNP	Genotypes			Alleles		OR (95% CI)	P-value
Maternal							
MTHFR C677T	CC	CT	TT	C	T	2.69 (1.35–5.34)	0.008*
Cases	33	11	6	77	23		
Controls	64	16	0	144	16		
MTHFR A1298C	AA	AC	CC	A	C	0.61 (0.36–1.03)	0.08
Cases	22	25	3	69	31		
Controls	26	40	14	92	68		
MTRR A66G	GG	AG	AA	G	A	1.02 (0.60–1.74)	1.00
Cases	17	33	0	67	33		
Controls	28	52	0	108	52		
GCP II C1561T	CC	CT	TT	C	T	1.89 (1.12–3.21)	0.03*
Cases	9	41	0	59	41		
Controls	37	43	0	117	43		
Paternal							
MTHFR C677T	CC	CT	TT	C	T	1.43 (0.52–3.93)	0.68
Cases	43	7	0	93	7		
Controls	72	8	0	152	8		
MTHFR A1298C	AA	AC	CC	A	C	1.25 (0.76–2.06)	0.50
Cases	8	36	6	52	48		
Controls	26	40	14	92	68		
MTRR A66G	GG	AG	AA	G	A	0.94 (0.54–1.62)	0.93
Cases	22	28	0	72	28		
Controls	33	47	0	113	47		
GCP II C1561T	CC	CT	TT	C	T	3.23 (1.76–5.93)	<0.0001*
Cases	16	34	0	66	34		
Controls	58	22	0	138	22		

*Statistically significant.

SNP = single nucleotide polymorphisms, OR = odds ratio, CI = confidence interval, GCP = glutamate carboxypeptidase, MTRR = methionine synthase reductase, MTHFR = methylene tetrahydrofolate reductase, NTD = neural tube defect.

Out of 80 control mothers, 37 (46.25%) were normal, 43 (53.75%) were heterozygous and none were homozygous mutant. The 1561T-allele frequencies were 41% and 26.9% in NTD case mothers and control mothers, respectively. This variant allele was associated with 1.9-fold risk for NTD.

Out of 50 NTD case fathers, 16 (32%) were normal, 34 (68%) were heterozygous and none were homozygous mutant for this polymorphism. Out of 80 control fathers, 58 (72.5%) were normal, 22 (27.5%) were heterozygous and none were homozygous mutant. The 1561T-allele frequencies were 34% and 13.75% in NTD case fathers and control fathers, respectively. This variant allele was associated with 3.2-fold risk for NTD.

MTRR 66A → G Out of 50 NTD case mothers, none were normal, 33 (66%) were heterozygous and 17 (34%) were homozygous mutant for MTRR 66A → G polymorphism. Out of 80 control mothers, none were normal, 52 (65%) were heterozygous and 28 (35%) were homozygous mutant. The 66G-allele frequencies were 67% and 67.5% in NTD case mothers and control mothers, respectively. This variant allele was not associated with any significant risk for NTD.

Out of 50 NTD case fathers, none were normal, 28 (56%) were heterozygous and 22 (44%) were homozygous mutant

for this polymorphism. Out of 80 control fathers, none were normal, 47 (58.75%) were heterozygous and 33 (41.25%) were homozygous mutant. The 66G-allele frequencies were 72% and 70.62% in NTD case fathers and control fathers, respectively.

Bivariate analysis

Pairwise analysis showed that 28% of NTD case mothers had co-segregation of MTHFR 677T-GCP II 1561T-variant alleles. None of the control mothers had this combination. Co-segregation of GCP II 1561T-MTRR 66G-variant alleles in mothers was associated with 3.9-fold risk for NTD. Co-segregation of MTHFR 677T-MTRR 66 G-variant alleles was not associated with statistically significant risk.

Paternal GCP II 1561T-MTRR 66G haplotype was associated with 5.6-fold risk for NTD. MTHFR 677T-MTRR 66G haplotype was not associated with a significant risk (Table 2).

Power calculations done for each statistically significant association showed sufficient sample size (Table 3).

Segregation of data according to type of defect revealed that maternal MTHFR 677T-variant allele was associated with 9-fold risk (95% CI: 3.77–21.55, $P < 0.0001$) for menin-

Table 2 Bivariate analyses between different genotypes.

Gene-Gene interaction	Combined genotype	Cases	Controls	OR (95% CI)	P-value
Maternal					
GCP 1561 MTHFR 677	C-C	6	21	Reference	
	C-T	3	16	0.66 (0.16–2.83)	0.88
	T-C	27	43	2.20 (0.80–5.96)	0.20
	T-T	14	0	Inf (10.87-Inf)	<0.0001*
GCP 1561/MTRR 66	C-G	9	37	Reference	
	C-A	0	0	ND	
	T-G	41	43	3.92 (1.70–8.99)	0.002*
	T-A	0	0	ND	
MTHFR 677/MTRR 66	C-G	33	64	Reference	
	C-A	0	0	ND	
	T-G	17	16	2.06 (0.93–4.56)	0.12
	T-A	0	0	ND	
Paternal					
GCP 1561/MTHFR 677	C-C	15	54	Reference	
	C-T	1	4	0.90 (0.13–6.64)	1.00
	T-C	28	18	5.60 (2.47–12.68)	<0.0001*
	T-T	6	4	5.40 (1.43–20.31)	0.04*
GCP 1561/MTRR 66	C-G	16	58	Reference	
	C-A	0	0	ND	
	T-G	34	22	5.60 (2.61–12.04)	<0.0001*
	T-A	0	0	ND	
MTHFR 677/MTRR 66	C-G	43	72	Reference	
	C-A	0	0	ND	
	T-G	7	8	1.47 (0.51–4.18)	0.67
	T-A	0	0	ND	

*Statistically significant.

Inf=infinite, OR=odds ratio, CI=confidence interval, GCP=glutamate carboxypeptidase, MTRR=methionine synthase reductase, MTHFR=methylene tetrahydrofolate reductase.

Table 3 Power calculations for statistically significant associations.

Genotype/alleles	Estimated proportion		Sample size = (α : 0.05, power: 80%)	
	Cases	Controls	Required	Actual
Maternal				
MTHFR 677TT	0.12	0.00	77	130
GCP II 1561CT	0.82	0.54	50	130
MTHFR 677T-allele	0.23	0.10	142	260
GCP II 1561T-allele	0.41	0.27	193	260
GCP II 1561T-MTHFR 677T	0.28	0.00	30	130
GCP II 1561T-MTRR 66G	0.82	0.54	50	130
Paternal				
GCP II 1561CT	0.68	0.28	29	130
GCP II 1561T-allele	0.34	0.14	81	260
GCP II 1561T-MTRR 66G	0.68	0.28	29	130

GCP = glutamate carboxypeptidase, MTRR = methionine synthase reductase, MTHFR = methylene tetrahydrofolate reductase.

gomyelocoele. Maternal and paternal GCP II 1561T-variant alleles were associated with 2.25- (95% CI: 1.12–4.50, $P < 0.05$) and 4.3 (95% CI: 2.01–9.09, $P < 0.001$) fold increased risk for anencephaly. No other statistically significant associations were observed between any other genotype and type of NTD.

Discussion

The current study showed that maternal MTHFR C677T and parental GCP II C1561T are genetically associated with NTD. Bivariate analyses showed significant interactions between MTHFR 677T/GCP II 1561T in mothers and GCP II 1561T/MTRR 66G in both parents. Our study is in agreement with the meta-analysis conducted by Botto and Yang, which showed 1.75 folds (95% CI: 1.14, 2.18) increased risk for NTD with MTHFR C677T polymorphism [2]. This study is the first to project GCP II C1561T genetic polymorphism in association to NTD. The risk associated with maternal MTHFR 677TT genotype could be due to thermolabile variant enzyme, which has enhanced propensity to dissociate into inactive monomers with subsequent loss in FAD binding capacity. This hampers the catalytic reduction of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate, leading to impaired methylation of homocysteine to methionine [23]. The lack of risk associated with MTHFR 1298A \rightarrow C polymorphism could be due to insignificant alteration in the specific activity of MTHFR as this polymorphism will not induce thermolability and is there in regulatory domain of the enzyme. The positive association with parental GCP II 1561C \rightarrow T could be due to low folate status in the parents as a result of impaired intestinal absorption of folates, which will affect the placental folate transport (maternal) and might induce sperm DNA damage (paternal). However, the role of this polymorphism in reducing the intestinal absorption of folate is still debated [1].

Segregation of data according to genotype showed that maternal MTHFR 677T-variant allele specifically increases

the risk for meningocele whereas GCP II 1561T-variant allele (maternal/paternal) increases risk for anencephaly. This might explain the failure of folic acid supplementation to prevent all NTDs suggesting that the etiology of defect at different sites might be different. Relton et al. have shown that MTHFR C677T and GCP II C1561T-variants significantly influence the risk of anencephalic pregnancy [13] whereas Dalal et al. have shown that maternal MTHFR C677T-variants significantly influence the risk for lower type of defects i.e., spina bifida [4].

Maternal MTHFR C677T and GCP II C1561T polymorphisms could result in significant depletion of functional folate levels and significant increase in plasma homocysteine levels, which affect the DNA synthesis and methylation pattern of the fetus and provide toxic environment for the fetus due to free radical generation as a result of auto-oxidation of homocysteine [17]. Paternal GCP II C1561T polymorphism could induce sperm DNA damage due to reduced folate availability for thymidylate synthesis thus increasing uracil misincorporation in DNA or it increases the chances of fetus inheriting this polymorphism especially when the mother also has this polymorphism.

Parental MTRR 66A $>$ G polymorphism alone was found to have no impact on the risk for NTDs. However, co-segregation of this polymorphism with GCP II 1561T-allele was found to be associated with significant risk both maternally and paternally. This shows strong evidence for epistatic interaction, where GCP II T-variant allele is acting as an epistatic allele and MTRR G-variant allele is acting as a hypostatic allele. The reason for such increased risk could be cumulative effect of defective folate absorption and defective activation of cobalamin II to cobalamin I, which hampers remethylation of homocysteine to methionine. This is consistent with the study of Wilson et al. showing increased risk for NTD in carriers of MTRR 66G allele when cobalamin levels were low [21], or in combination with MTHFR 677C $>$ T mutant genotype. More recently Zhu et al. reported an association between the G allele and NTD risk in a US population [24].

Both maternal and paternal MTHFR 677T- and GCP II 1561T-variant alleles were found to have synergetic interaction in increasing the risk for NTD. The reason for such synergetic effect could be cumulative effect of defective folate absorption and defective synthesis of 5-methyl tetrahydrofolate.

Lower availability of folate, 5-methyl tetrahydrofolate and cobalamin (I) will result in low methionine synthesis, which, in turn, affects the synthesis of universal methyl donor, S-adenosyl methionine. During pregnancy, the requirement of circulating folate and methionine increases significantly, as maternal to fetal transport of these precursors is essential during embryo development both for DNA synthesis and cellular methylation [15]. These polymorphisms adversely affect this process thereby resulting in hypomethylation. Altered methylation profile of genes could have major consequences during the neurulation phase in which gene expression of the different genes involved in the closure of the neural tube are following each other in a cascade event [10].

This study indicates significant gene-gene interactions between different loci and thus, highlights the importance of multiple loci in folate pathway for predicting the risk of NTD. There is need to investigate gene-nutrient interactions also for more precise risk prediction. The limitations of this study are its sample size and lack of fetal/proband samples to investigate maternal-fetal gene-gene interactions.

In summary, maternal *MTHFR* 677TT genotype and parental *GCP II* 1561CT genotype were found to elevate the risk for meningomyelocele and anencephaly, respectively, in South Indian subjects. Significant gene-gene interactions have been observed in the study group. *MTHFR* 677×*GCP II* and *GCP II*×*MTRR* combinations in both parents were found to significantly increase the risk for NTD in this population. Parental genotype combinations in different folate pathway loci seem to predict susceptibility for NTDs more precisely than genotypes at individual loci. These data clearly emphasize the need for multi-locus analyses in the risk prediction of such multi-factorial disorders.

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